A Combination of CNTF and BDNF Rescues *rd* Photoreceptors but Changes Rod Differentiation in the Presence of RPE in Retinal Explants

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Purpose. To gather information regarding the combination of ciliary neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF), compared with the individual factors when used as a treatment to retard photoreceptor cell loss in *rd* mouse retina explants and to investigate the observation that the retinal pigment epithelium (RPE) influences rod differentiation by this treatment.

Methods. Postnatal day (PN)2 or PN7 control and *rd* mouse retinas were grown with attached retinal pigment epithelium (RPE). The explants were kept in culture up to PN28. During this culture period CNTF, BDNF, CNTF+BDNF, or vehicle were continuously administered to the culture medium. The nontrophic factors cyclosporin A and *N*-CBZ-aspartic acid-glutamic acid-valine-aspartic acid-fluoromethyl ketone (z-DEVD-fmk) were also used. The number of photoreceptor nuclei remaining in the outer nuclear layer (ONL) was analyzed in hematoxylin and eosin–stained sections. Rod- and cone-specific antibodies were used to determine identity and state of differentiation of the photoreceptors.

Results. Compared with vehicle treatment, BDNF or CNTF resulted in 1.4- or 2-fold more surviving cell rows in the ONL, respectively. However, when CNTF and BDNF were applied together, surviving ONL cell counts in the *rd* explants were approximately 3 times those in vehicle-treated explants. In the presence of CNTF or CNTF+BDNF, opsin and arrestin expression in rods was decreased compared with rods without attached RPE. Cyclosporin A and z-DEVD-fmk did not show rescue of *rd* photoreceptor cells.

Conclusions. CNTF or BDNF treatment of *rd* retinal explants delays photoreceptor cell loss to some extent. However, when these agents are combined, photoreceptor rescue is much more effective. The quenching of opsin and arrestin expression caused by treatment suggests that simultaneous with rod rescue, rod differentiation is depressed. Regarding retinal degeneration, the results from the selective inhibitors of apoptosis rank the CNTF+BDNF combination treatment as the most consistent and effective experimental pharmacologic intervention currently available. (*Invest Ophthalmol Vis Sci.* 2001;42: 275–282)

Rod photoreceptor degeneration in the *rd* mouse is the consequence of a mutation in the gene for the β-subunit of the cyclic guanosine monophosphate (cGMP) phosphodiesterase effector protein.[1] The mutation leads to toxic accumulation of the second messenger cGMP in the cell body, which in turn, causes cell death through apoptosis.[2–4] A mutation in the same gene has been found in human forms of autosomal recessive retinitis pigmentosa (RP)[5–7] making the *rd* mouse retina an ideal model for experimental analysis of human retinal dystrophies. To create a more accessible tissue for experimentation, we have developed an organ culture procedure for neonatal mouse retina.[8] An equivalent rabbit neonatal retina organ explant culture has also been developed.[9] Over the years it has been reported that retinal explants derived from normal and mutant mice cultured according to this procedure display much of their in vivo phenotype in a comparable timetable, albeit a little slower. Photoreceptor cell bodies in areas with overlying retinal pigment epithelium (RPE) show outer segment disc material, whereas in areas without attached RPE, only inner segment, but no outer segment, material is observed.[10–12] Therefore, this culture procedure is suitable to screen the effect of different agents on photoreceptor rescue in the *rd* retina in a reliable and effective manner. It is assumed that consistent photoreceptor rescue in culture indicates therapeutic potential for in vivo studies.

Various neurotrophic factors have been tested for their ability to slow retinal cell loss.[13] Some have been found to be effective when injected into the vitreous and subretinal space,[14] when applied to dissociated cultures,[15] or as a supplement to transplants.[16] We have shown that a combination of basic fibroblast growth factor (FGF2) and nerve growth factor (NGF), but not these agents on their own, retards *rd* photoreceptor cell loss in organ culture.[12] A key emerging theme is the requirement for combinations of specific agents when designing an effective pharmacologic intervention for neuroprotection. Regarding protection of photoreceptor cells against hereditary degeneration, recent data have indicated that another pair of neurotrophic factors, ciliary neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF), are effective in delaying cell loss in *rd* retinal tissue in culture.[17] The combination of CNTF and BDNF has demonstrated powerful neuroprotection of developing retinal ganglion cells and in motor neuron disease as well.[18,19]

A purpose of our study, from which we report the results, was to investigate the combined effect of CNTF and BDNF on photoreceptor survival in detail. We wanted to gather sufficient information regarding dosage, reliability, duration of effect, and relative effectiveness of the combination treatment compared with application of the individual factors. For this, our neonatal retinal explant procedure has been used. While examining the immunolabeled sections, we noted a drug-induced decrease in opsin immunoreactivity when in contact with the RPE. This phenomenon was investigated further because this observation appealed to reports that CNTF blocks photoreceptor differentiation in vitro.[20] The results presented herein enable a better understanding and interpretation of effects displayed by the neurotrophic factors and the role of the RPE.

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Materials and Methods

Animal Treatment and Tissue Culture Conditions

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The organ culture was described in detail previously.[8,10–12] Briefly, pigmented *rd* (*rd/rd*) and congenic control (+/+) C3H mouse pups were decapitated at approximately 48 hours (postnatal day [PN] 2) or 7 days (PN7) after birth and the eyes removed. After cleansing with 70% ethanol, the eyes were incubated in basal medium supplemented with 1.2% proteinase K (Sigma, St. Louis, MO) at 37°C for 15 minutes. The anterior segment, vitreous body, and sclera were removed and the retina flat mounted with the photoreceptor-side down on a cellulose filter attached to a polyamide grid. Most explants were cultured with RPE, a few without RPE, and a few as a sandwich. In a sandwich explant, part of the retinal tissue was folded so that photoreceptors were facing up as well as down during culturing.

Retinas were incubated in 1.2 ml of R16 medium (Gibco, Gaithersburg, MD) with 10% or 2% fetal bovine serum (FBS; Gibco). For vehicle treatment either no further agents or dimethyl sulfoxide (DMSO) was added to the culture medium. The latter was used as a control for agents dissolved in DMSO-containing solution. For the neurotrophic factor treatment groups, the serum medium was supplemented with 50 ng/ml or 10 ng/ml of either recombinant rat CNTF or recombinant human BDNF (Saveen; ReproTech, Rocky Hill, NJ) or both. For the other treatment groups the medium was supplemented with either cyclosporin A (CsA; 25 μg/ml) or the *N*-carbobenzoxy amino acid sequence *N*-CBZ-aspartic acid-glutamic acid-valine-aspartic acid-fluoromethyl ketone (z-DEVD-fmk; 0.67 μg/ml). The CsA was purchased from Novartis–Sandoz (Arnhem, The Netherlands; provided in a 50-mg/ml intravenous infusion solution). z-DEVD-fmk was purchased from Calbiochem-Novabiochem (Nottingham, UK) and kept in DMSO stock solutions. For this report CsA and z-DEVD-fmk were neither tested at multiple doses nor in 2% FBS.

Statistical Analysis and Immunocytochemistry

All retinal explants were cultured up to PN28. The tissue was fixed in 4% paraformaldehyde, infiltrated with 25% sucrose in Sörensen's phosphate buffer, cryosectioned (8–10 μm) and stained using hematoxylin and eosin (H&E). These sections were viewed and either accepted into or rejected from the explant population. Accepted explants were assigned a neutral tag (consecutive numbers). Exclusion criteria included a dead explant or presence of fibroblast growth. Links between fibroblast growth and a particular treatment were not analyzed and therefore cannot be excluded. To draw inferences about the accepted population, 4 samples (explants) from each category (number of explants per category, at least 13) were taken at random and the number of rows in a vertical column of the outer nuclear layer (ONL) counted. Two experienced observers, who were uninformed about each other's results, collected the data.

Variability between counts was negligible. For cultured explants ONL column counts was used as a measure, because we and others[21] have observed that any distortion of the tissue tends to affect the thickness of the ONL more than the number of somata in a vertical column. The number of four explants was decided to be adequate, serving as a good approximation unless indicated otherwise by statistical analysis. Counts were from central regions of sections with flat ONL, because the explants flatten off or show higher degrees of rosettes at the periphery. For reasons explained later, data from PN2 and PN7 explants of the same category were pooled. The highest number of rows of nuclei in the ONL (for maximum effect) was noted, and comparisons were made using one-way analysis of variance (ANOVA) at the 5% significance level, followed by Fisher's protected least-significant difference post hoc comparisons. It was not possible to section explants according to retinal horizontal or vertical planes. Therefore, it could not be determined whether counts were from superior or inferior parts of the central retina. The cells in the inner nuclear layer (INL) and ganglion cell layer were not analyzed.

The identity and state of differentiation of individual cell types in the ONL were further investigated by immunocytochemistry. Four antibodies were used: a polyclonal opsin antibody (AO, 1:15,000), a polyclonal arrestin antibody (1:30,000), a monoclonal green cone antibody (COS-1, 1:2,000), and a monoclonal blue cone antibody (OS-2, 1:2,000).[12,22] Secondary antibodies conjugated with biotin that was reacted with avidin-horseradish peroxidase (avidin-HRP) and diaminobenzidine (DAB; Vector, Burlingame, CA) detected the bound antibodies. All histochemical and immunocytochemical reactions were examined and reproduced with either a photomicroscope (Axiophot: Zeiss, Oberkochen, Germany) or a microscope (BX60; Olympus, Lake Success, NY) equipped with Optronics image analysis hardware operated by image analysis software (Micro Image; Olympus) on a desktop computer (Presario; Compaq, Houston, TX).

Table 1. Protective Effect of CsA or DEVD on *rd* Cell Loss in Culture

	Vehicle	CsA (25 μg)	DEVD (16.7 μg)
rd 10% FBS	2.3 ± 0.2	2.5 ± 0.1	2.4 ± 0.2
rd 2% FBS	2.4 ± 0.1		
+/+ 10% FBS	7.8 ± 0.1	8.0 ± 0.1	8.4 ± 0.2
+/+ 2% FBS	8.4 ± 0.1		

Data are means ± SEM. ONL counts in *rd* and +/+ explants cultured in 10% or 2% serum-containing medium (FBS) up to PN28. Vehicle, CsA, and DEVD represent continuous vehicle, cyclosporin A, and z-DEVD-fmk treatment, respectively. CsA was applied in a concentration of 25 μg/ml culture medium. z-DEVD was applied in a concentration of 16.7 μg/ml culture medium. The counts from vehicle, CsA, z-DEVD-fmk were in the same range indicating no protective effect. Data were collected from four explants.

Results

Observations on Explant Age and Serum Levels

The reason for using PN2- and PN7-aged tissues was to look for a possible critical period for the treatment's effectiveness during the first stage of the *rd* degenerative process. The explants were all analyzed at PN28 to create maximum differences between degenerating and surviving ONLs. No difference between PN2 and PN7 explants was observed. Explants with or without RPE were used to check for a possible role of the RPE on ONL cell counts. ONL cell counts from explants without RPE were less stable than from explants with RPE attached, and the following quantitative analysis was therefore derived from explants with RPE attached. Consequently, PN2 and PN7 explants with RPE attached of each category were pooled for statistical analysis. High- and low-serum–containing media were used to capture interference by other factors possibly present in the serum. The results are displayed in the second column (vehicle) of Table 1. Vehicle treatment of *rd* explants in 10% FBS medium maintained 2.3 ± 0.2 (mean ± SEM) photoreceptor rows, whereas in 2% FBS medium 2.4 ± 0.1 rows were observed in the ONL. Vehicle treatment of +/+ explants in 10% FBS medium resulted in 7.8 ± 0.1 rows of cells in the ONL, whereas explants cultured in 2% FBS showed 8.4 ± 0.1 rows of nuclei. Statistical analysis of our entire data set (Tables 1, 3) showed instances of significant differences between high- and low-serum–containing media, indicating that low-serum medium caused better survival of photoreceptor cells. However, the effect caused by low-serum–containing medium was never more than 1.5-fold of the effect produced by high-serum medium.

TABLE 2. Influence of High Serum on Protective Effect of CNTF, BDNF, and CNTF+BDNF on *rd* Cell Loss in Culture

	Vehicle	CNTF (50 ng)	CNTF (10 ng)	BDNF (50 ng)	BDNF (10 ng)	CNTF+BDNF (50 ng)	CNTF+BDNF (10 ng)
rd 10% FBS	2.3 ± 0.2	4.3 ± 0.2	4.3 ± 0.1	2.9 ± 0.2	2.8 ± 0.1	6.5 ± 0.2	7.0 ± 0.4
rd 2% FBS	2.4 ± 0.1	4.5 ± 0.1	4.3 ± 0.2	3.2 ± 0.2	3.2 ± 0.1	7.0 ± 0.4	6.9 ± 0.1
+/+ 10% FBS	7.8 ± 0.1	8.0 ± 0.2	7.6 ± 0.3	8.6 ± 0.1	8.1 ± 0.2	8.4 ± 0.1	8.1 ± 0.4
+/+ 2% FBS	8.4 ± 0.1	7.8 ± 0.4	7.6 ± 0.3	8.4 ± 0.1	8.5 ± 0.2	8.4 ± 0.2	8.3 ± 0.2

Data are means ± SEM. ONL counts in *rd* and +/+ explants cultured in 10% or 2% serum-containing medium up to PN28. Each factor was applied in a concentration of 10 ng/ml or 50 ng/ml culture medium. Counts from explants cultured in low- or high-serum medium were in the same range. Data were collected from four explants.

CNTF or BNTF

Results from experiments using only CNTF or BDNF are summarized in Table 2. Addition of 50 ng/ml CNTF to a 10% FBS medium maintained 4.3 ± 0.2 rows in the ONL of *rd* explants. The same treatment of these explants developed in 2% serum-containing medium produced 4.5 ± 0.1 rows of cells in the ONL. CNTF (10 ng/ml) in 10% FBS medium showed 4.3 ± 0.1 rows of photoreceptor cells, whereas 10 ng/ml of this survival factor in 2% FBS medium resulted in 4.3 ± 0.2 rows in the ONL of *rd* explants. Application of 50 ng/ml CNTF to control explants growing in 10% serum-containing medium led to survival of 8.0 ± 0.2 rows of photoreceptor cells, whereas the same treatment of these explants cultured in 2% serum-containing medium resulted in 7.8 ± 0.4 rows of cells in the ONL. CNTF (10 ng/ml) displayed an ONL thickness of 7.6 ± 0.3 and 7.6 ± 0.3 rows of photoreceptor nuclei in +/+ explants in 10% and 2% serum, respectively.

Application of 50 ng/ml BDNF to a 10% FBS medium resulted in 2.9 ± 0.2 rows of nuclei in the ONL of *rd* explants. A similar treatment of these explants growing in 2% FBS medium produced 3.2 ± 0.2 rows of cells in the ONL. BDNF (10 ng/ml) in 10% serum-containing medium showed 2.8 ± 0.1 rows of photoreceptor cells, whereas 10 ng/ml of this survival factor supplemented to 2% serum-containing medium resulted in 3.2 ± 0.1 rows of photoreceptor nuclei in the ONL of *rd* explants. Addition of 50 ng/ml BDNF to +/+ explants growing in 10% FBS medium led to survival of 8.6 ± 0.1 rows of photoreceptor cells, whereas the same treatment of these explants cultured in 2% FBS medium produced 8.4 ± 0.1 rows of cells in the ONL. BDNF (10 ng/ml) in 10% and 2% serum-containing media resulted in an ONL thickness of 8.1 ± 0.2 and 8.5 ± 0.2 rows of photoreceptor nuclei in control sections, respectively.

CNTF+BNTF

Tables 2 and 3 summarize the results obtained when the retina was subjected to CNTF+BDNF during development in culture up to PN28. Administration of 50 ng/ml CNTF+BDNF to a 10% serum-containing medium maintained 6.5 ± 0.2 rows in the ONL of *rd* explants. A similar treatment of these explants put in 2% serum-containing medium resulted in 7.0 ± 0.4 rows of cells in the ONL. CNTF+BDNF (10 ng/ml) in 10% FBS medium showed 7.0 ± 0.4 rows of photoreceptor cells, whereas after addition of 10 ng/ml of this survival factor in 2% FBS medium 6.9 ± 0.1 rows were observed in the ONL of *rd* explants. Supplement of 50 ng/ml CNTF+BDNF to +/+ explants cultivated in high serum levels led to survival of 8.4 ± 0.1 rows of photoreceptor nuclei, whereas the same treatment to control explants cultured in low serum levels resulted in 8.4 ± 0.2 rows of cells in the ONL. CNTF+BDNF (10 ng/ml) displayed an ONL thickness of 8.1 ± 0.4 and 8.3 ± 0.2 rows of photoreceptor nuclei in control explants cultured in 10% and 2% FBS media, respectively.

TABLE 3. *rd* Photoreceptor Cell Rescue by CNTF+BDNF in Culture

	Vehicle	CNTF+BDNF (50 ng)	CNTF+BDNF (10 ng)
rd 2% FBS	2.4 ± 0.1	7.0 ± 0.4	6.9 ± 0.1
+/+ 2% FBS	8.4 ± 0.1	8.4 ± 0.2	8.3 ± 0.2
rd 10% FBS	2.3 ± 0.2	6.5 ± 0.2	7.0 ± 0.4
+/+ 10% FBS	7.8 ± 0.1	8.4 ± 0.1	8.1 ± 0.4

Data are means ± SEM. ONL counts in *rd* or +/+ explants cultured in 2% or 10% serum-containing medium up to PN28. The difference in ONL counts between control- and neurotrophic factor-treated *rd* explants was significant. Data were collected from four explants.

Comparisons of CNTF, BNDF, and CNTF+BDNF

Figure 1 displays histologic images from the untreated *rd* control (Fig. 1A), followed by the *rd* retinal explant treated with 10 ng/ml CNTF (Fig. 1B), BDNF (Fig. 1C), or CNTF+BDNF (Fig. 1D), when cultured in a 2% serum-containing medium. Companion +/+ explants are depicted in Figures 1E through 1H. In both serum conditions, all the CNTF treatments had a statistically significant rescue effect ($P < 0.001$) in *rd* explants when compared with vehicle treatment. On average, the number of rows in the *rd* ONL was twofold in CNTF-treated explants compared with vehicle treatment. ONL counts in CNTF-treated *rd* retinas were not statistically different between high- and low-serum conditions. There were no significant differences when 10 ng/ml or 50 ng/ml CNTF was applied. Compared with BDNF, CNTF had a significant rescue effect in 10%, but not in 2%, FBS medium. Comparisons between BDNF and vehicle treatments of *rd* explants showed a statistically significant ($P < 0.05$; except 10 ng/ml BDNF in both serum concentrations) rescue of photoreceptors by BDNF. No statistically significant differences were found between high and low concentrations of BDNF when tested in *rd* explants. High- and low-serum–containing media produced similar effects when BDNF was tested. In *rd* explants, all cases of CNTF+BDNF treatment resulted in a significant ($P < 0.001$) rescue effect in comparison with vehicle treatment. On average, CNTF+BDNF produced three times as many rows in the ONL of *rd* explants when compared with vehicle treatment. Again, the low dose of the combination treatment had already produced the maximum effect. CNTF+BDNF ONL cell counts were twice those of BDNF and 1.5 times that of CNTF.

Within the control (+/+) explant group, low-serum–containing medium had a statistically significant positive effect over high-serum–containing medium ($P < 0.05$). Similarly, 50 ng/ml BDNF and 50 ng/ml CNTF+BDNF produced significantly more counts when compared with vehicle-treated 10% FBS control explants.

Figure 2 illustrates part of the statistical comparisons between treatments by showing the interaction bar plots

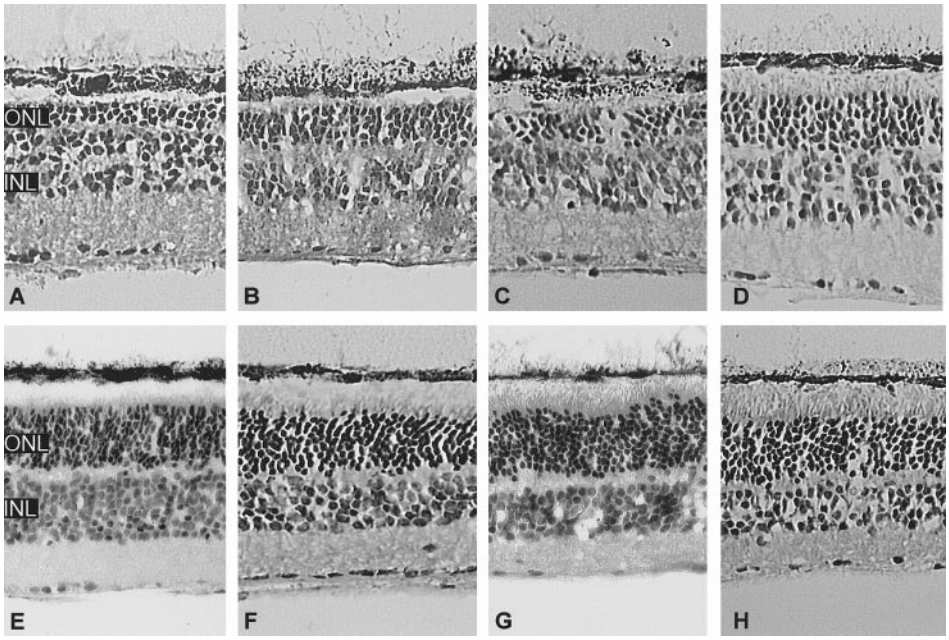

FIGURE 1. Light microscopic photomicrographs of mouse retinal explants with RPE cultured in 2% serum-containing medium up to PN28. (**A**) An *rd* explant treated with vehicle, (**B**) 10 ng/ml CNTF, (**C**) 10 ng/ml BDNF, or (**D**) 10 ng/ml CNTF+BDNF. (**E**) A +/+ explant treated with vehicle, (**F**) 10 ng/ml CNTF, (**G**) 10 ng/ml BDNF, or (**H**) 10 ng/ml CNTF+BDNF. The large number of cells surviving in the ONL can be seen in (**D**).

(mean ± SEM) for the vehicle-treated +/+ and *rd* explants and the 10 ng/ml neurotrophic factor drug treatments of *rd* explants.

Opsin and Arrestin Immunoreactivity in CNTF and CNTF+BDNF Treated Explants with RPE Attached

After having observed the spatial differences in opsin immunoreactivity within sandwich explants (Fig. 3A), we adapted this method for analyzing the role of the RPE in connection with treatment. Opsin expression in the genotypic normal (+/+) explants appeared to vary consistently, depending on whether the tissue was cultured in either CNTF alone or the combination of CNTF and BDNF. Photoreceptors in a normal explant devoid of RPE and cultured in a CNTF+BDNF medium, displayed opsin immunoreactivity on both sides of the sandwich (Fig. 3B). Photoreceptors in a similar explant with RPE attached at one side showed delay of opsin expression by those rods adjacent to the RPE (Fig. 3C). This result was neither dependent on serum concentration nor specific to high or low levels of appropriate neurotrophic factors. Opsin immunolabeling in the genotypically normal explants maintained in BDNF, CsA, or z-DEVD-fmk was, regardless of the presence of RPE, equivalent to that displayed in Figure 3B (not shown). To check for a possible selectivity of the differential opsin expression sections were also labeled with an arrestin antibody.

Figure 4A depicts a sandwich explant illustrating the more pronounced arrestin immunoreactivity by rescued *rd* photoreceptors without RPE. Figure 4B shows an image taken from a section of an identically treated age-matched (PN2) common *rd* explant. Note the differential arrestin immunoreactivity when the RPE was not attached, suggesting that the differential opsin staining is not limited to this molecule. Opsin labeling in similar *rd* tissue (Figs. 4C, 4D) shows the same phenomenon, suggesting that modulation of photoreceptor proteins by the RPE is not limited to tissue of a certain genotype.

To complete the demonstration of the effect by the CNTF+BDNF treatment, photographs of immunolabeled normal (+/+) explants are presented in Figure 5. Arrestin immunoreactivity was ambiguous in treated (Fig. 5A) explants, whereas untreated (Fig. 5B) tissue consistently showed clear staining. The observation for the opsin (Figs. 5C, 5D) immunoreactivity is that in the treated normal explants only a minor subpopulation of rodlike cells were immunopositive (Fig. 5C), whereas the untreated normal explants displayed an opsin distribution pattern usually seen in the explant culture—that is, labeling of the entire ONL.

Green cone immunolabeling was not observed. Blue cone immunostaining was encountered, as described previously. All control labeling procedures were devoid of immunoreactivity.

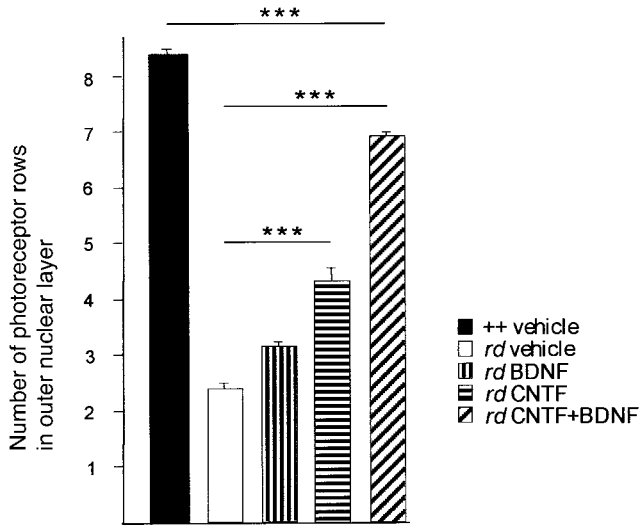

FIGURE 2. Interaction bar plots for ONL counts as coded. Values are plotted on the *x*-axis; bars represent mean ± SEM. ***$P < 0.001$.

Apoptosis Inhibitors

Both CsA and z-DEVD-fmk failed to block *rd* photoreceptor cell loss in 10% FBS medium. Other observations at the light microscopy level included some photoreceptor nuclei dislocated to the subretinal space. Photoreceptor cell bodies in areas with overlying RPE showed outer segment disc material, whereas in areas without attached RPE, only inner segments were observed.

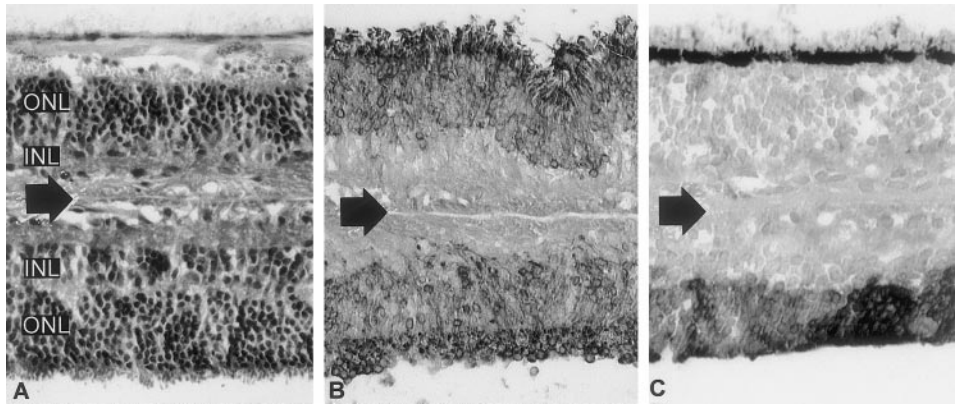

FIGURE 3. Light microscopic photomicrographs of mouse sandwich retinal explants cultured up to PN28. The top flap of the explant was placed on the membrane carrier during culture. This side was attached to the RPE, when present. The bottom flap was facing the medium–air interface. *Arrow*: Border between the retinal flaps. (**A**) Histology of +/+ sandwich explant. (**B**) A +/+ sandwich explant cultured without RPE and treated with 50 ng/ml CNTF+BDNF in 10% FBS medium. Photoreceptors showed rod opsin immunostaining at both sides of the explant. (**C**) A +/+ sandwich explant cultured with RPE and treated with 50 ng/ml CNTF+BDNF in 10% FBS medium. The pigmented RPE is visible at the *top* of (**C**). Rod opsin immunoreactivity was much reduced in photoreceptors facing the RPE compared with photoreceptors facing away and located in the bottom flap of the sandwich explant.

DISCUSSION

The scope of this study was to gather information regarding dosage, reliability, duration of treatment, and relative effectiveness of CNTF+BDNF combination treatment compared with application of the single proteins when applied to *rd* retina.

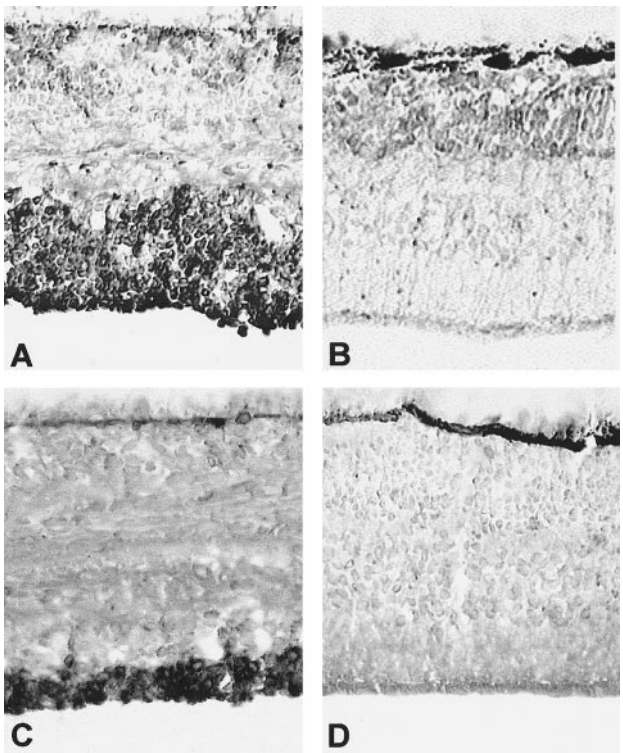

FIGURE 4. Light microscopic photomicrographs of mouse *rd* retinal explants cultured up to PN28 in CNTF+BDNF medium. Orientation is the same as in Figure 3. All specimens had RPE attached. Arrestin labeling: (**A**) Sandwich explant. Photoreceptors facing away from the RPE were strongly labeled compared with those adjacent to the RPE. (**B**) Normal aligned explant: Photoreceptors displayed reduced immunostaining. Opsin labeling: (**C**) Sandwich explant. Photoreceptors facing away from the RPE were strongly labeled, and there was an absence of immunoreactivity in photoreceptors adjacent to the RPE. (**D**) Normal aligned explant: Immunostaining was absent in the photoreceptors.

For interpretation of the results, we first positioned the neonatal retinal organ culture procedure by comparing it with age-matched in vivo data that have been published extensively and in detail.[23] In vivo PN28 retinas of the *rd* and congenic control (+/+) mouse have, respectively, 1 row and 10 to 12 rows of photoreceptor nuclei in the ONL. In our vehicle-treated organ explants of the same genotype and age the numbers were approximately two and nine, respectively. Data about the ultrastructure of the tissue have been reported previously.[8] Regarding synthesis of photoreceptor-specific pro-

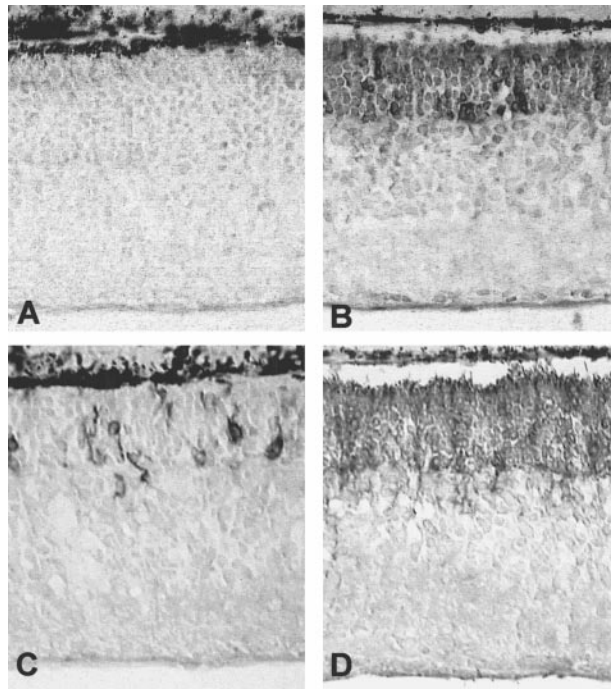

FIGURE 5. Light microscopic photomicrographs of mouse (+/+) retinal explants cultured up to PN28 in CNTF+BDNF medium (**A**, **C**) and without addition of neurotrophic factors (**B**, **D**). Orientation is the same as in Figure 4. All retinas had RPE attached. (**A**, **B**) Arrestin immunolabeling treated explants (**A**) was reduced compared with that in untreated specimens (**B**). (**C**, **D**) Opsin immunoreactivity labeling was strong in rod photoreceptors cultured without neurotrophic factors (**D**) compared with the few stained rod photoreceptors in the treated explants (**C**).

teins in control explants, we observed no deviations from earlier findings.[10,11] Thus, our retinal organ culture model is a good approximation, but still an approximation, of its in vivo counterpart.

If these properties are taken into account and proper controls are used, this technique can be used to generate valid information regarding altered numbers of ONL cells in response to treatment. The organ culture method can be compared with culturing many different cell types simultaneously. Their nutritional requirements may vary from one cell to another. We therefore elected to add serum, containing proteins and polypeptides, carbohydrates, hormones, and vitamins that were unknown to the experimenter but often necessary for more demanding cell lines. When performance between the high- and low-serum–containing media is compared, the evidence does not suggest major interference by unknown serum factors in our system. The observation that in 2% FBS medium slightly more photoreceptor cells survived than in 10% FBS medium is a sign that high serum levels should be avoided. This corresponds to data published by Gaur et al.[24] Because all the ONL counts have been performed on retinas with RPE attached (this produced more stability) we cannot say whether the presence of RPE is necessary to produce a beneficial effect by the applied treatments.

Neurotrophic Factor–Mediated RPE Effects on Photoreceptor-Specific Protein Expression

Some investigators have reported that CNTF on its own can significantly block apoptosis.[14,25] In our system CNTF has maintained approximately 4.5 ONL rows, which, when left untreated, would have decreased to 2.4 rows in the *rd* retina by PN28. Thus, CNTF was able to rescue *rd* retinal photoreceptors on its own, as published earlier.[26] BDNF application, when compared with CNTF, resulted in less delay of *rd* cell loss, which is in agreement with findings by others.[14] CNTF+BDNF, however, has shown to be the most potent photoreceptor rescue, maintaining approximately seven ONL rows in the *rd* retina. This is a synergistic effect from both individual proteins and is generated when the tissue is continuously exposed to 10 ng/ml of the combination therapy. In this case we have not visualized whether the neurotrophic factor treatment affects either the rate of cell death or mitosis. Alterations of either can lead to cell gain, but other studies using either identical treatment or other neurotrophic factors have reported arrest of disease and no increase in mitosis.[13–19] Currently, we are investigating the time during which the first exposure to the combination treatment is effective and whether subsequent intermittent applications can maintain photoreceptor rescue up to PN28.

The present result is in accordance with data demonstrating that simultaneous administration of CNTF and BDNF significantly inhibits motor neuron loss in the Wobbler mouse in contrast to these agents alone.[19] It is also similar to the earlier finding that FGF2, combined with NGF but not applied individually, saves *rd* photoreceptors in culture.[12] Synergistic interactions between various neurotrophic factors are now a recurring phenomenon and have been superior over the individual agents.[27,28] As yet, it remains unresolved how CNTF+BDNF combination therapy, for which the ligand receptors are not expressed by the photoreceptors themselves but are located on the neighboring retinal RPE and Müller glia cells,[29–33] transmit their signal to the photoreceptor cell nuclei. The use of Trk B and CNTFR-α antibodies may provide important clues about the mechanism.

In this study, we also observed that CNTF or CNTF+BDNF causes a reduction in opsin and arrestin immunoreactivity in photoreceptor cell bodies that overlay the RPE. This effect is stronger for opsin than for arrestin expression. Using rat dissociated rods CNTF also blocks rod differentiation, but this capability of CNTF diminishes with maturation of the cells.[20] We agree with this concept; however, our interpretation is that this phenomenon is selective and incomplete. This issue is important, because it suggests that promoting rod survival by this type of drug therapy may compromise function at the same time. Using electroretinography, a similar phenomenon has been noted recently by Gargini et al.[34] Loss of function is one of the first serious side effects to be considered when neurotrophic factors are used to rescue retinal cells. An explanation for the observed side effects by CNTF or CNTF+BDNF may be found in the kinds of interactions engaged by these neurotrophic factors and the RPE–retina–interphororecaptor matrix (IPM) complex. The hypothesis is that these interactions promote rod photoreceptor cell survival but delay rod photoreceptor differentiation. Strong support comes from Layer et al.[35] who, looking for effects of the RPE on histogenesis of the avian retina in vitro, have come to the conclusion that RPE extends cell proliferation, whereas differentiation is much delayed. They found that the effects of RPE on rod differentiation occur without the influence of neurotrophic factors. In our retinal organ culture, however, the strong delay in rod differentiation by the RPE occurs only under the influence of CNTF and BDNF. This indicates that in the mouse the action of these neurotrophic factors is direct or indirect through the RPE.

FGF2 is released by the RPE and likely also by Müller cells in response to CNTF[36] and is able to directly stimulate photoreceptors.[37] Therefore, FGF2 is a strong candidate to function as a messenger between the neurotrophic factors acting on the RPE–retina–IPM complex on the one hand and the photoreceptors on the other hand. In contrast, it has also been discovered that FGF2 antibodies inhibit the differentiation of neural retina without an effect on apoptosis or proliferation.[38] In a previous study in which we analyzed FGF2+NGF rescue of *rd* photoreceptor cells in organ culture, an RPE modulation of opsin immunoreactivity was not noticed. This suggests some functional differences between elevated FGF2 levels due to RPE secretion and those due to added recombinant bovine FGF2. In any case, to minimize any impact on rod function, a safety and efficacy evaluation of survival factors including retinal metabolic parameters should be conducted before these drugs are used in clinical trials.

No Effect of Apoptotic Pathway Inhibitors on *rd* Cell Loss In Vitro

We have begun evaluating CsA as a potential drug for inhibiting photoreceptor cell loss through apoptosis. CsA blocks release of cytochrome *c* from mitochondria, which prevents activation of caspase 9, acting upstream of caspase 3, the proximal caspase in the apoptotic pathway.[39,40] When *rd* retina was exposed to 25 μg/ml CsA in 10% FBS medium, rescue from cell loss was not observed. The dosage used in the current study was the same as the one used to stop thyroid-induced apoptotic regression of the tadpole tail.[40] However, in another study using T cells, apoptosis has been effectively blocked using a 1-μg/ml concentration of CsA.[41] Therefore, the dosage used in our experiment may have been too high, inducing interference with the inhibition of photoreceptor cell apoptosis. Söderpalm et al.[42] have reported recently that z-VAD-fmk, a broad-spectrum caspase blocker, effectively stops retinoic acid-induced photoreceptor cell death in vitro. Caspase-3 is activated in transgenic rats with opsin mutation S334ter, and photoreceptor loss through apoptosis can be delayed by intraocular injection with z-DEVD-fmk.[43] z-DEVD-fmk is a caspase-3–selective inhibitor. We did not observe z-DEVD-fmk–induced rescue of mouse *rd* cells in vitro. In a preliminary study, Chong et al.[44]

have injected several caspase inhibitors into the vitreous of the retinal degeneration slow (*rds*) and *rd* mice. They found only an effect of the caspase-3 inhibitor III (Ac-DEVD-CMK) in the *rds* mouse. No significant effects have been found either from the other treatments or in the *rd* mouse. This is similar to our experience, which supports the conclusion by Chong et al.[44] that the protective effect by the caspase-3 inhibitors is ambiguous or better, selective to specific mutations. Because little information is currently available about retinal specificity, proper dosage, when to begin therapy, and best delivery regimen for caspase inhibitors, further studies are needed. In any case, the emerging picture from these apoptosis blockers is relevant to the neurotrophic factor issue. From the experimental drug treatments for inherited retinal degeneration currently under investigation, CNTF, and BDNF rank as the most potent and consistent broad-spectrum treatment when assayed in vitro. This warrants taking the next step: an in vivo comparison of this combination therapy against alternatives like diltiazem.[45] It is from these systematic in vitro screenings followed by in vivo testing that true successful pharmacologic therapies for retinal degeneration will emerge.

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